p62^{cdc23} of *Saccharomyces cerevisiae*: A Nuclear Tetratricopeptide Repeat Protein with Two Mutable Domains

ROBERT S. SIKORSKI, WILLIAM A. MICHAUD, AND PHILIP HIETER*

Department of Molecular Biology and Genetics, The Johns Hopkins University
School of Medicine, 725 North Wolfe Street, Baltimore, Maryland 21205

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CDC23 is required in *Saccharomyces cerevisiae* for cell cycle progression through the G₂/M transition. The CDC23 gene product contains tandem, imperfect repeats, termed tetratricopeptide repeats, (TPR) units common to a protein family that includes several other nuclear division CDC genes. In this report we have used mutagenesis to probe the functional significance of the TPR units within CDC23. Analysis of truncated derivatives indicates that the TPR block of CDC23 is necessary for the function or stability of the polypeptide. In-frame deletion of a single TPR unit within the repeat block proved sufficient to inactivate CDC23 in vivo, though this allele could rescue the temperature-sensitive defect of a cdc23 point mutant by intragenic complementation. By both in vitro and in vivo mutagenesis techniques, 17 thermolabile cdc23 alleles were produced and examined. Fourteen alleles contained single amino acid changes that were found to cluster within two distinct mutable domains, one of which encompasses the most canonical TPR unit found in CDC23. In addition, we have characterized CDC23 as a 62-kDa protein (p62^{cdc23}) that is localized to the yeast nucleus. Our mutagenesis results suggest that TPR blocks form an essential domain within members of the TPR family.

Temperature-sensitive mutants which terminate division at discrete stages (termed *cdc* mutants) can serve as valuable tools in studies of the cell cycle (for a review, see reference 27). Particularly applicable for investigation of the chromosome segregation process are those mutants that arrest after DNA replication but prior to anaphase. Several *cdc* mutants (*cdc13*, *cdc16*, *cdc17*, *cdc20*, and *cdc23*) arresting at this G₂/M stage have been isolated in *Saccharomyces cerevisiae*. At semipermissive temperatures, mutations in *cdc13* and *cdc17* increase both the rate of chromosome loss and the rate of recombination (9), phenotypic characteristics expected of genes required for DNA synthesis or repair (28). Mutations in *cdc16*, *cdc20*, and *cdc23* perturb the fidelity with which chromosomes are transmitted during mitosis but have little or no effect on DNA recombination (9). This latter group, therefore, identifies genes that are excellent candidates for encoding structural or regulatory components of the mitotic machinery.

The wild-type *CDC23* and *CDC16* genes have been cloned previously (16, 36), and analysis of their predicted amino acid sequences has revealed an underlying structural relationship. Both proteins contain multiple copies, mostly in tandem at the carboxy termini, of an imperfect 34-amino-acid repeat unit termed TPR (tetratricopeptide repeat [37]). To date, TPR-like units have been discovered in 14 genes from six different organisms, which include fungi, bacteria, insects, and humans (for reviews, see references 6, 15, and 39). Genetic studies have shown that half of these genes are required for completing early stages of mitosis.

On the basis of the analysis of the amino acid composition of the TPR, it has been proposed that the TPR units may adopt an amphipathic α -helical conformation (13, 37). The resulting hydrophobic surfaces could, in principle, direct protein-protein or protein-membrane associations important

for executing early stages in mitosis (13). Using a genetic assay, we have shown that two TPR proteins, *CDC23* and *CDC27*, are capable of interacting in vivo (39). To date, however, no biochemical activity has yet been assigned to any TPR protein or to the TPR units that they contain.

In this report, we have used mutagenesis to address the role of TPR units in executing the function performed by *CDC23* in vivo. Through the study of deletion mutations, we have shown that the C-terminal repeats in *CDC23* are required for biologic activity. Analysis of point mutations found in 17 thermolabile *cdc23* alleles revealed the existence of two mutable domains, one at the amino terminus and one within the TPR at the carboxyl terminus of the protein. Antisera to *CDC23* were used to show that, like all other TPR *CDC* proteins analyzed thus far, the *CDC23* polypeptide is concentrated within the yeast nucleus. Our data, together with structural comparisons of three homologous TPR proteins, encoded by *CDC27*, *nuc2⁺*, and *BimA*, from different organisms, suggest that tandem arrays of TPR units may represent a discrete and perhaps modular structural domain.

MATERIALS AND METHODS

Strains and growth media. Table 1 lists the genotypes and sources of the yeast strains used. Routine yeast cultures were maintained on YPD medium (2% glucose, 1% yeast extract, 2% peptone) containing 2% agar. Yeast transformants were grown on SD medium plus required auxotrophic supplements (35). The permissive temperature for thermolabile mutants was 25°C; the nonpermissive temperature was 37°C.

DNA manipulations. Standard techniques were used for DNA cloning and restriction enzyme analysis (7, 23). A rapid boiling method was used to characterize putative clones (14). *Escherichia coli* DH5 α [F⁻ *endA1*, *hsd17(rk⁻ mk⁺)* *supE44 thi-1 recA gyrA96 relA1* Δ (*argF-lacZ*) *U169* Φ 80 *lacZ* Δ

* Corresponding author.

TABLE 1. Yeast strains

Strain	Description or genotype	Source or reference
4533-7-2	α <i>cdc23-1 ura1 ade2 ade3 his7 leu2 can1 sap3</i>	Lee Hartwell
5011	a <i>cdc23-2 cdc24-1 ade ade2 gal1 his7 lys2 tyr1 ura1</i>	Lee Hartwell
E73	a <i>cdc23-4 ade1 ade2 gal1 his7 lys2 tyr1 ura1</i>	Lee Hartwell
E6-2	a <i>cdc23-6 ade1 ade2 gal1 his7 lys2 tyr1 ura1</i>	Lee Hartwell
4522-28	a <i>cdc28-1 ura1 ade2 ade3 his7 leu2 can1 sap3</i>	Lee Hartwell
YPH275	a <i>lys2-801 ade2-101 ura3-52 his3-Δ200 trp1-Δ1 leu2-Δ1</i> CFVII(RAD2.d.YPH275) TRP1 SUP11	Hieter collection
YPH940	α <i>lys2-801 ade2-101 ura3-52 his3-Δ200 trp1-Δ1 leu2-Δ1</i>	This study
YPH941	YPH275 transformed with cut pRS11 (<i>cdc23::HIS3</i>)	This study
YPH942	YPH940 transformed with pRS20 (<i>CDC23</i> on episome)	This study
	α <i>cdc23::HIS3 lys2-801 ade2-101 ura3-52 his3-Δ200 trp1-Δ1 leu2-Δ1</i> CFVII(RAD2.d.YPH275) TRP1 SUP11 containing pRS20	
YPH943	a <i>cdc23::HIS3 lys2-801 ade2-101 ura3-52 his3-Δ200 trp1-Δ1 leu2-Δ1</i> containing pRS20	This study
YPH944	Diploid product of mating between YPH942 and YPH943	This study
YPH500	α <i>lys2-801 ade2-101 ura3-52 his3-Δ200 trp1-Δ63 leu2-Δ1</i>	Hieter collection
YPH945	α <i>lys2-801 ade2-101 ura3-52 his3-Δ200 trp1-Δ63 leu2-Δ1 cdc23-37</i>	This study
GY32	a <i>ura3-52 trp1-Δ63 leu2-Δ1 prbl-1122 pep4-3 prcl-407</i>	Gary Yellen
7434-2	Tetraploid strain of <i>S. cerevisiae</i>	Doug Koshland

M15] from Bethesda Research Laboratories was used for recombinant DNA manipulations. Yeast transformations were performed by the method of Ito et al. (17). Restriction enzymes were purchased from New England Biolabs and Bethesda Research Laboratories.

General plasmid constructs. Table 2 lists and references all plasmids used in this study. pRS20 contains an 8-kb *SalI* fragment of yeast genomic DNA (isolated from a λ phage clone that spans the wild-type *CDC23* locus) ligated into the unique *SalI* site of the *URA3*-based *CEN* vector YCp50 (31). This plasmid provided the original isolate of the *CDC23* gene reported by Sikorski et al. (37).

pRS22 is a *LEU2*-based *CEN* vector made by ligating a *HindIII* fragment of YCp19 containing *CEN4* and *ARS1* into the unique *HindIII* site of pLK1. The latter plasmid contains a 2.2-kb *SalI*-*XhoI* fragment of the yeast *LEU2* gene inserted into the *SalI* site of pUC8 (39a).

pRS34 was made by ligating a 4.2-kb *SpeI*-*SalI* fragment

carrying *CDC23* (isolated from pRS20) into the *SalI* and *BamHI* sites of pRS22. Prior to ligation, the *SpeI* and *BamHI* sites were both made blunt ended by the action of Klenow DNA polymerase. The *BamHI* site was regenerated in the process of subcloning.

pRS90 was made by cloning a 2.6-kb *BamHI*-*XbaI* fragment from pRS34 containing a minimal, wild-type *CDC23* gene and flanking sequences into the *BamHI* and *XbaI* sites of pBLUESCRIPT-KS+ (Stratagene).

pRS144 was made by subcloning the 2.6-kb fragment of *CDC23* from pRS90 into the *BamHI* and *XbaI* sites of the *TRP1/CEN/ARS* vector pRS314 (38).

pRS148 is a deletion derivative of pRS90 designed to remove all ATG sequences 5' to the initiator methionine codon of the *CDC23* open reading frame (ORF). pRS90 was partially digested with the enzyme *BclI*, which cleaves at three sites within *CDC23*. Full-length linear products were purified, digested completely with *BamHI*, and recircularized. A clone deleted to -19 with respect to the initiator ATG was identified by nucleotide sequencing.

Metabolic labelling with ^{35}S . One millicurie of Tran- ^{35}S -label (a mixture of [^{35}S]methionine and [^{35}S]cysteine; ICN Biochemicals) was added to 5 ml of a yeast culture in SD medium grown to an optical density at 600 nm of 1.0 (approximately 2×10^8 cells). After incubation at 30°C for 1.5 h, the cells were pelleted, washed with water, and transferred to a 1.5-ml screw-capped tube (Sarstedt Co.). Five hundred microliters of hot lysis buffer (1% sodium dodecyl sulfate [SDS], 150 mM NaCl, 20 mM NaPi [pH 7.4] at 65°C) was added, and the cells were disrupted by vigorous agitation (Vortex) for 5 min at room temperature in the presence of a 0.5-ml volume of glass beads (0.5-mm diameter; Sigma) and then incubated for 5 min at 100°C. After centrifugation at $15,000 \times g$, the supernatant was used as labelled extract for immunoprecipitation.

Immunoprecipitation. Immunoprecipitations were performed at 4°C with constant mixing. Fifty microliters of labelled extract was diluted into 500 μl of Nonidet P-40 (NP-40) buffer (1% NP-40, 150 mM NaCl, 2 mM EDTA, 50 mM Tris-HCl [pH 8.0]) containing 1 mM phenylmethylsulfonyl fluoride. To remove nonspecific precipitating material, 50 μl of protein A agarose (50% suspension; Calbiochem) was added for 1 h and then removed by centrifugation. Three microliters of anti-*CDC23* sera or control preimmune sera

TABLE 2. Plasmids

Plasmid	Relevant sequence	Source or reference
YCp50	<i>ARS1 CEN4 URA3</i>	31
pRS20	YCp50 + <i>CDC23</i>	This study
pLK1	pUC8 <i>LEU2</i>	Mitch Smith (University of Virginia)
pRS22	<i>ARS1 CEN4 LEU2</i>	This study
pRS34	pRS22 + <i>CDC23</i>	This study
pRS90	pBLUESCRIPT + <i>CDC23</i>	This study
pRS314	<i>ARSH4 CEN6 TRP1</i>	38
pRS144	pRS314 + <i>CDC23</i>	This study
pRS148	<i>CDC23</i> ORF (T3 promoter)	This study
pATH3	Amino terminus of <i>TrpE</i>	19a
pRS133	pATH3 + <i>CDC23</i> ORF	This study
pRS192	pRS314 + <i>cdc23-Δ1</i>	This study
pRS190	pRS314 + <i>cdc23-Δ2</i>	This study
pRS191	pRS314 + <i>cdc23-Δ3</i>	This study
pRS179	pRS314 + <i>cdc23-Δ4</i>	This study
pRS193	pRS314 + <i>cdc23-Δ5</i>	This study
YIp5	<i>URA3</i>	40a
pRS11	YIp5 + <i>CDC23</i>	This study
pRS4	<i>cdc23::HIS3</i> transplacement vector	This study
pRS112	<i>cdc23-37</i> transplacement vector	This study

was added to the resulting supernatant. After 1 h, 50 μ l of protein A-agarose was again added and incubated for 1 h. The agarose beads were then washed four times with 500 μ l of RIPA buffer (150 mM NaCl, 1.0% NP-40, 2 mM EDTA, 50 mM Tris-HCl [pH 8.0]). To solubilize bound material, 100 μ l of 2 $\times$ Laemmli sample buffer (20) was added to the pellets and heated to 100°C for 5 min. Fifty microliters of this material was subjected to SDS-polyacrylamide gel electrophoresis (PAGE) (1). After Entensify (New England Nuclear Co.) was added, the gel was dried and placed in contact with XAR film (Kodak) at -70°C.

In vitro protein synthesis. *CDC23* cRNA was produced in vitro from the T3 promoter of *Xba*I-linearized pRS148 by using T3 RNA polymerase and a commercially available kit (Stratagene). One unit of inhibitACE (5'-3' Co.) RNase inhibitor was added per reaction. Approximately 2 μ g of the resulting uncapped cRNA (shown to be of the expected size by agarose gel electrophoresis) was used in a protein synthesis reaction in a rabbit reticulocyte lysate system (Promega Biotec) in the presence of [³⁵S]methionine (1,200 Ci/mM; Amersham).

***TrpE-CDC23* fusion protein.** To manipulate the *CDC23* coding sequence, a *Bgl*II linker (5'-CAGATCTG-3') was inserted into the *Sna*BI site just 3' to the predicted initiator ATG codon of *CDC23* in the plasmid pRS34. A 4.0-kb *Bgl*II to *Sal*I fragment from the resulting recombinant was subcloned into the *Bam*HI and *Sal*I sites of the bacterial expression vector pATH3 (4) to make pRS133. Upon induction with indole acrylic acid in *Escherichia coli*, pRS133 produced a *TrpE-CDC23* fusion protein containing 33 kDa of *TrpE* and all but 13 amino-terminal amino acids of *CDC23*. The *TrpE-CDC23* fusion protein was highly degraded in many bacterial hosts, and a full-length product could be produced only in the strain FZ-392 (a gift of Rich Janis, Abbott Laboratories). The fusion protein was purified from bacterial extracts by virtue of its insolubility in 1.5 M NaCl-12 mM MgCl₂ (19).

Antibody production. Insoluble protein (approximately 80% *TrpE-CDC23*) from induced FZ-392 (+pRS133) was separated by preparative SDS-PAGE (20). The fusion protein band was identified by staining with CuSO₄ (21), excised, dried, and rehydrated in Freund's adjuvant. Approximately 200 μ g of fusion protein in a suspension of Freund's complete adjuvant injected subcutaneously into two New Zealand White rabbits was followed by 100- μ g boosts at 3-week intervals.

Synthetic 15-mer peptides were made corresponding to amino-terminal (PEP1; AATELSRWKLYGSSK) and carboxy-terminal (PEP2; TSQEIEEARMLAREC) regions within the predicted *CDC23* protein ORF. Four milligrams of peptide was dissolved in 3 ml of 100 mM NaPi (pH 6.8) and mixed with 8 mg of KLH dissolved in 1 ml of 10 mM NaOH. Eight microliters of 50% glutaraldehyde (EM grade) was then added, and cross-linking was allowed to proceed for 4 h. The reaction was terminated by extensive dialysis against phosphate-buffered saline (PBS; 150 mM NaCl, 20 mM NaPi [pH 7.35]). Initially, 200 μ g of conjugated peptide was injected as described above and then followed by several boosts of 100 μ g each.

Anti-PEP2 sera was affinity purified before its use in immunofluorescence microscopy. Briefly, 10 mg of peptide was dissolved in 20 ml of 100% ethanol, and 3 ml of Affi-Gel 10 matrix (50% slurry in isopropanol; Bio-Rad) was then added. Coupling was terminated after 12 h at room temperature by the addition of 300 μ l of 1 M ethanolamine. Crude rabbit antisera was bound to a column containing the PEP2-

Affi-Gel matrix. Elution was accomplished with both high and low pH washes as described previously (8). The binding activity of the eluate was evaluated by Western blotting (immunoblotting) against bacterial extracts containing the *TrpE-CDC23* fusion protein.

Western blotting. Total cellular proteins were extracted from mid-log-phase yeast cultures by disruption with glass beads in the presence of detergent as described previously (8). After SDS-PAGE, the proteins were transferred to nitrocellulose membranes and processed as described previously (8, 41). The anti-peptide sera was used at a 1:2,000 dilution. [¹²⁵I]-protein A was used for visualization of the bound antibodies.

Immunofluorescence microscopy. Yeast cells were grown to mid-log phase in rich medium (YPD), fixed with 3.7% formaldehyde, and processed for antibody staining as described previously (5). The primary antibody was affinity-purified anti-PEP2 sera at a 1:200 dilution in Tris-buffered saline (TBS; 200 mM NaCl, 50 mM Tris-HCl [pH 7.4]) plus 3% bovine serum albumin. The secondary antibody was fluorescein-conjugated goat anti-rabbit sera (Cappel Labs) used at a 1:1,000 dilution.

Sequencing *CDC23* mutations. Oligonucleotides complementary to the wild-type *CDC23* sequence were synthesized (Applied Biosystems) to serve as primers for dideoxy sequencing (32) by using the Sequenase enzyme (United States Biochemical Corp.). Nine 17-mer oligonucleotides (5'-TATT AGCGAGCGGAAAA; AAACACACTCTTTAGCC; ATTT CTATCGTGGGATA; CTGGAGTCTTATGAAAT; CGAT GCATTGCTTTTAA; TTATGTTACCGCAGAAA; ATGTA TTTCCGTCGAGC; CTTGCACTTTGAAACCT; TGTGGA AGAATTCTGG-3') were made in the sense orientation of *CDC23* such that they were spaced evenly every 220 nucleotides throughout the coding sequence beginning at nucleotide -69. Shuttle vectors carrying the *cdc23* alleles produced through in vitro mutagenesis and plasmid shuffling were recovered in *E. coli* from the appropriate yeast strains and used directly as sequencing templates (11).

In order to obtain the nucleotide sequence of previously existing, chromosomal *cdc23* alleles, the polymerase chain reaction (PCR) (24) was first used to amplify DNA representing the various mutant coding sequences. Genomic DNA was isolated from yeast strains 4533-7-2 (*cdc23-1*), 5011 (*cdc23-2*), E73 (*cdc23-4*), E6-2 (*cdc23-6*), and 4522-28 (*CDC23*) (gifts of Lee Hartwell). Approximately 1 μ g of DNA was used in each PCR (AmpliTaQ; Cetus) (denaturing, 94°C for 1 min; annealing, 37°C for 2 min; extension, 72°C for 5 min) with two oligonucleotides (5'-GCAGACCAAGTAC TGCGGTACTCAC-3' and 5'-CGGTTTCATCCTTCTGCCA TGGTCAC-3') designed to amplify a 2.1-kb fragment of *CDC23*. The PCR products were digested with *Nhe*I and *Nde*I to release a 1.8-kb fragment of the 1.9-kb coding sequence. Mutant *cdc23* genes were reconstructed in vitro (to contain wild-type 5' and 3' flanking sequences) by cloning these *Nhe*I-*Nde*I fragments into the *Nhe*I-*Nde*I sites of the plasmid pRS144, replacing the wild-type *CDC23* coding sequence in this plasmid. The entire coding sequence of each mutant was obtained (as described above), and all mutations identified were confirmed by sequencing one other independent isolate.

***CDC23* truncations and internal deletion mutations.** These *cdc23* alleles were all derived from the 2.6-kb *Spe*I-*Xba*I fragment carrying a minimal clone of *CDC23* (37) by manipulating the coding sequence at restriction enzyme recognition sites. pRS192 (*cdc23-Δ1*) was made by ligating a synthetic 8-bp *Bgl*II linker (5'-CAGATCTG-3') into the unique

*Xmn*I site of the *CDC23* ORF of pRS144. This creates a frameshift mutation which truncates the natural *CDC23* coding sequence at Glu-566. pRS190 (*cdc23-Δ3*) and pRS191 (*cdc23-Δ2*) were each made by partially digesting pRS144 with *Hind*III, blunt-ending the sites by using Klenow polymerase, and ligating them on *Bgl*II linkers. In both cases the ligation product creates a TGA termination codon in the *CDC23* reading frame, and the natural *CDC23* coding sequence in these alleles is truncated at Ala-491 ($\Delta 3$) and Ala-528 ($\Delta 2$). pRS179 (*cdc23-Δ4*) was constructed by digesting *CDC23* with *Eco*RI, blunt-ending with Klenow polymerase, and reclosing. The frameshift in this allele truncates the natural coding sequence at Asn-379. pRS193 (*cdc23-Δ5*) is a derivative of pRS144 containing a small, in-frame deletion between the two internal *Hind*III sites within the *CDC23* coding sequence (37).

***CDC23* null allele and *CDC23* tester strains.** A 2.1-kb *Eco*RI fragment containing the carboxyl terminus of *CDC23* (as well as 0.9 kb of DNA 3' to the *Xba*I site of the 2.6-kb *Spe*I-*Xba*I fragment) was subcloned from the 8-kb phage insert into the integrating vector YIp5. The resulting plasmid, pRS11, was digested completely with *Xba*I and partially with *Hind*III (the site in *CDC23* furthest from *Xba*I was cut) and ligated to an *Eco*RI-*Bam*HI fragment containing the yeast *HIS3* gene (after all ends were made flush with Klenow polymerase). This transplacement vector, pRS4, was linearized with *Eco*RI and transformed into the strain YPH275. A transformant, YPH940, was selected and shown by Southern blot analysis to be heterozygous for the truncated (*cdc23::HIS3*) allele.

To make a *cdc23* null allele "tester" strain, the 8-kb *Sal*I *CDC23* fragment was first cloned into the *Sal*I site of YCp50 to make pRS20. This plasmid was then introduced into YPH940 to make YPH941, which was then sporulated. Two His⁺/Ura⁺ spores, which were inviable in the presence of 5-fluoroorotic acid (5-FOA), were chosen from four spore tetrads and designated YPH943 and YPH942. These were mated to form a diploid strain, YPH944, which was isolated by micromanipulation.

To make a *cdc23* temperature-sensitive allele tester strain carrying a defined point mutation, the *cdc23-37* allele was removed from the in vitro mutagenized construct (after recovery in *E. coli*) as a *Bam*HI (genomic *Spe*I site) to *Sal*I fragment and ligated into the *Bam*HI and *Sal*I sites of the *URA3*-based integrating vector pRS24 (similar in structure to pRS306 [38]), making pRS112. After linearization at the unique *Eco*RI site within the *cdc23* gene, pRS112 was transformed into the haploid strain YPH500 (38). A Ura⁻ segregant of this transformant, YPH945, which evicted the wild-type *CDC23* gene, was then selected by its growth on 5-FOA and its temperature sensitivity.

RESULTS

Construction of a *cdc23* null allele: *CDC23* is essential for viability. To initiate genetic studies of *CDC23*, a null allele (*cdc23::HIS3*) was constructed to delete the carboxyl-terminal 136 amino acids of *CDC23*, including TPR-8 and TPR-9 (see reference 37 for TPR nomenclature). The null allele was introduced at one of the *CDC23* loci of a diploid strain by transformation, with the yeast *HIS3* gene linked to facilitate the identification of transformants and follow meiotic segregation of the mutant allele. Sporulation of the resulting diploid strain, YPH940 (*CDC23/cdc23::HIS3*), yielded two viable and two inviable spores in 24 tetrads, and all viable spore colonies were His⁻. This confirms that *CDC23* is an

essential yeast gene. Microscopic examination of the inviable spores revealed that they had germinated, entered the cell cycle, and arrested uniformly in the first or second division with a large-budded morphology. Thus, complete lack of *CDC23* protein produces a *cdc* terminal phenotype similar to that of thermolabile alleles at a nonpermissive temperature (see below).

A plasmid shuffle test for *CDC23* function: repeats in the tandem block are essential. To probe the functional significance of TPR in *CDC23*, we first constructed a set of mutant alleles which produced truncated and internally deleted forms of the *CDC23* protein and tested whether these mutants could complement the growth defect of the *cdc23::HIS3* allele. Our initial strategy for making the truncations was to simply truncate the *CDC23* gene; however, the 3' untranslated region of *CDC23* proved to be essential for function (data not shown). We chose instead to mutate the *CDC23* ORF through the insertion of frameshift mutations or termination codons as described in Materials and Methods. These constructions leave the 3' untranslated region intact and change only slightly the overall structure of the mRNA.

The functional status of each mutant allele was tested in a papillation assay using a yeast strain in which we could easily "uncover" the *cdc23* null allele. This method is a variation of the plasmid shuffle technique commonly used for generating yeast mutants (2, 36). We constructed a haploid yeast strain, YPH942, that contains the *cdc23::HIS3* null allele integrated into the genome and a wild-type copy of the *CDC23* gene carried on an episome, in this case the *URA3*-based YCp50 vector. Cells which lose an episome containing the *URA3* gene (with concomitant loss of the wild-type *CDC23* gene) can be selected directly by growth on the drug 5-FOA, a compound that kills Ura3⁺ cells. Since *CDC23* is essential for growth, cells which lose this plasmid (an event that occurs about 2% per cell division) are inviable. Thus, YPH942 is unable to generate Ura3⁻ papillae on 5-FOA. However, Ura3⁻ segregants will remain viable if they contain a second plasmid that provides *CDC23* function. Papillation of YPH942 on 5-FOA after transformation with a second test plasmid indicates that the second plasmid carries a functional (for vegetative growth) *CDC23* gene.

The truncated and internally deleted *cdc23* mutations were introduced into YPH942 on a single-copy *CEN-LEU2* vector, and the resulting strains were tested for their ability to grow on media containing 5-FOA. The results are presented in Fig. 1. As expected, the control (pRS34 + wild-type *CDC23*) supports growth on 5-FOA while the vector alone does not. The *cdc23-Δ1* mutant allele lacking amino acid residues beyond 567 supports the growth of YPH942 on 5-FOA at a rate only slightly lower than that of wild-type *CDC23*. The Ura3⁻ cells carrying this allele are normal in size and morphology as judged by phase-contrast microscopy. These results demonstrate that the final 61 amino acids of *CDC23*, which do not contain a TPR motif, are dispensable for essential mitotic functions.

The next truncated allele in the series, *cdc23-Δ2*, which lacks residues beyond amino acid 528, does not rescue the *cdc23* null mutation. The additional 38 amino acids removed in this truncation compared with *cdc23-Δ1* represent all of TPR-9 and 7 amino acids of TPR-8. These TPR residues appear to be critical for *CDC23* function or stability. Mutations that further truncate the *CDC23* polypeptide, *cdc23-Δ3* or *cdc23-Δ4*, also do not support YPH942 growth in the presence of 5-FOA.

We also analyzed a small in-frame deletion of 37 amino

FIG. 1. Functional analysis of deletion derivatives of *CDC23*. Plasmid constructs carrying the various deletions were introduced into YPH942 (a strain designed for plasmid shuffling) or YPH945 (a strain containing an integrated copy of the *cdc23-37* temperature-sensitive [ts] allele). *CDC23* function was assessed by growth on 5-FOA for YPH942 or growth at 37°C for YPH945. The *cdc23-Δ5* mutation complemented the growth defect of YPH945 at 33°C but not 37°C. Note that the *cdc23-37* allele alone will not support growth above 30°C. The sequence of the repeat shaded black best fits the overall TPR consensus (37). CDC23^{wt}, wild-type *CDC23* gene.

acids (residues 491 to 527) within the *CDC23* coding sequence, *cdc23-Δ5*. This deletion removes a single repeat unit containing every element of the TPR consensus sequence, the highly conserved regions A and B of TPR-8, and the proline containing region of TPR-7 (see Fig. 1A of reference 37 for the amino acid sequence). When tested in the papillation assay, *cdc23-Δ5* behaves as all other nonfunctional alleles in being unable to complement the growth defect of *cdc23::HIS3*.

A complementation test for *CDC23* function: intragenic complementation between a deletion and point mutant. The various *cdc23* alleles were also assayed for their ability to complement the temperature-sensitive growth defect of a *cdc23-37* allele. A tester strain (YPH945) was constructed by replacing the wild-type *CDC23* locus in a haploid yeast strain with the *cdc23-37* mutation. An evaluation of YPH945's growth at a spectrum of temperatures showed that this particular mutant exhibits a sharp transition to inviability and *cdc* arrest between 28 and 30°C. The individual *cdc23* mutant alleles were then introduced into YPH945 on single copy *CEN* plasmid vectors, and the ability of the resulting transformants to grow at different temperatures was examined (Fig. 1).

The only *cdc23* alleles which fully complement YPH945 are *cdc23-Δ1* and *CDC23*^{wt}. This is consistent with the plasmid shuffle data on these alleles and again shows that the terminal 61 residues of *CDC23* are dispensable. Truncations which removed amino acids in the block of repeats were uniformly nonfunctional for complementation at any of the tested temperatures. The *cdc23-Δ5* internal deletion allele complemented the conditional lethality of YPH945 at 30°C. Note that *cdc23-Δ5* alone is incapable of providing sufficient *CDC23* function for growth at 30°C (see above). Therefore, such growth must be the result of intragenic complementation between the TPR deletion and point mutant alleles.

In vitro mutagenesis of *CDC23*. We next chose in vitro mutagenesis of the entire *CDC23* coding sequence as an approach to probing the role of individual amino acid residues in performing the biological function of *CDC23*. The *CDC23* gene was mutagenized with hydroxylamine in vitro and introduced by transformation into YPH942. After plas-

FIG. 2. Thermolabile alleles of *CDC23*. A YCp vector carrying the wild-type (wt) *CDC23* gene was mutagenized in vitro in the presence of hydroxylamine, and 17 temperature-sensitive alleles were isolated after plasmid shuffling. Cultures of YPH942 harboring plasmid-borne derivatives of the 10 tightest *cdc23* temperature-sensitive alleles (including two alleles produced by in vivo mutagenesis; see Materials and Methods) were grown to saturation at the permissive temperature and 10-fold serial dilutions were performed. By using a pronged inoculator, aliquots of each dilution were placed on rich medium and incubated at the indicated temperatures.

mid shuffling (36) of 8,500 individual yeast transformants selected at 25°C, 15 strains were found to be temperature sensitive for growth at 37°C (Fig. 2). The mutagenized plasmids were recovered from the individual yeast strains and introduced back into YPH942; in all cases the plasmids conferred a temperature-sensitive phenotype after plasmid shuffling. To further demonstrate linkage of the temperature-sensitive mutations to the *CDC23* ORF, we subcloned all of the mutated coding sequences (see Materials and Methods) into a vector containing unmutagenized flanking sequences of *CDC23*. After showing that the resulting plasmids indeed all carried temperature-sensitive alleles of *CDC23* by the plasmid shuffling assay, the entire subcloned ORF of each was sequenced and the mutations were identified (Table 3). The 15 sequences represented 13 distinct alleles; two sequences were represented twice and presumably were derived from sibling clones of *E. coli*.

The majority of *cdc23* temperature-sensitive alleles produced in vitro were shown to contain single amino acid substitutions (Fig. 3A). The remaining alleles were double mutants (Fig. 3B). As a group, the single missense mutations were distributed nonrandomly throughout the *CDC23* polypeptide such that they clustered within two "mutable domains," one toward the N terminus and the other within the repeat block near the C terminus. To exclude the possibility that these two regions were simply preferred targets for hydroxylamine induced mutations, we examined the nature of mutations in *cdc23* temperature-sensitive alleles produced through an alternate mutagenesis technique.

In vivo mutagenesis of *CDC23*. During the original screen for *cdc* mutations in *S. cerevisiae*, several alleles of *cdc23* were isolated after mutagenesis with *N*-methyl-*N'*-nitro-*N*-nitrosoguanidine (8a). We purified total genomic DNA from

TABLE 3. Mutations found in thermolabile alleles of *cdc23*

Allele ^a	Nucleotide ^b	Codon	Amino acid
<i>cdc23-1</i>	G→A (1,416)	GGC→GAC	G→D (472)
<i>cdc23-2</i>	A→G (1,316)	CAC→CGC	H→R (439)
<i>cdc23-4</i>	C→T (281)	ACG→ATG	T→M (94)
<i>cdc23-6</i>	G→A (368)	AGT→AAT	S→N (123)
<i>cdc23-37</i>	G→A (1,192)	GAA→AAA	E→K (398)
<i>cdc23-39</i>	G→A (1,210)	GCG→ACG	A→T (404)
<i>cdc23-40</i>	G→A (308)	CGA→CAA	R→Q (103)
	G→A (1,212)	GCG→GCA	(Silent)
	G→A (1,718)	GTA→ATA	V→I (573)
<i>cdc23-41</i>	C→T (340)	CCA→TCA	P→S (114)
	C→T (888)	ATC→ATT	(Silent)
<i>cdc23-44</i>	G→A (238)	GGC→AGC	G→S (80)
<i>cdc23-47</i>	G→A (438)	GGG→GAG	G→D (213)
	G→A (1,749)	TGG→TGA	W→TERM (583)
<i>cdc23-49</i>	G→A (916)	GAG→AAG	E→K (306)
	C→T (977)	CCC→CTC	P→L (326)
<i>cdc23-50</i>	G→A (115)	GCG→ACG	A→T (39)
<i>cdc23-51</i>	G→A (253)	GAG→AAG	E→K (85)
<i>cdc23-52</i>	C→T (278)	TCT→TTT	S→F (93)
<i>cdc23-53</i>	C→T (341)	CCA→CTA	P→L (114)
<i>cdc23-54</i>	G→A (125)	GGT→GAT	G→D (42)
<i>cdc23-56</i>	C→T (1,453)	CTT→TTT	L→F (485)
<i>cdc23-57</i>	Same as <i>cdc23-41</i>		
<i>cdc23-59</i>	Same as <i>cdc23-39</i>		

^a The *cdc23-1*, -2, -4, and -6 mutations are those that differ from the wild-type allele of *CDC23* isolated from the strain A364a. See Materials and Methods for details.

^b The numbers in parentheses refer to the number of nucleotides mutated within *CDC23* with respect to the A residue of the initiator ATG.

four of these mutants (*cdc23-1*, *cdc23-2*, *cdc23-4*, and *cdc23-6*) and used a PCR-based strategy to obtain plasmid clones of the mutant *cdc23* genes. In order to identify nucleotide changes responsible for the temperature-sensitive phenotype associated with these *cdc23* alleles, we first determined the sequence of the wild-type *CDC23* gene present in the A364a background of the parent strain. This was necessary for comparison purposes since the published sequence of *CDC23* was obtained from a different *S. cerevisiae* strain, S288C (37). The wild-type *CDC23* gene from the A364a strain 4522-28 was found to contain six polymorphisms with respect to the S288C *CDC23* sequence. All were located in the third position of codons and thus did not alter the predicted amino acid sequence. The changes were S288C-A364a, TTC-TTT (nucleotide [nt] 354), AAC-AAT (nt 744), GTT-GTC (nt 786), ACT-ACC (nt 1011), GCC-GCT (nt 1347), and GCC-GCT (nt 1371).

Sequence analysis of the cloned *cdc23* alleles revealed that each contained a single nucleotide change and amino acid substitution compared with the A364a wild-type *CDC23* allele (Table 3). The four mutations were all located within the two mutable domains identified by our previous in vitro mutagenesis (Fig. 3A, starred residues). This result indicates that the mutable domains of *CDC23* are not simply preferred targets for the mutagen or method used (hydroxylamine [in vitro] or nitrosoguanidine [in vivo]) and suggests that these regions represent essential functional domains within the polypeptide.

Identification of p62^{*cdc23*}. To identify the polypeptide product encoded by *CDC23*, we generated antisera by immunization with synthetic peptides or a *TrpE-CDC23* fusion protein. Anti-peptide sera directed against antigenic determinants at the N terminus (PEP1) and C terminus (PEP2) of *CDC23* both recognized a single 62-kDa species by Western

A.

B.

C.

FIG. 3. Schematic location of amino acid substitutions found in thermolabile alleles of *CDC23*. (A) All single residue changes were mapped to one of two mutable domains. The alleles denoted with an asterisk were produced by in vivo mutagenesis with nitrosoguanidine. All other alleles were produced by in vitro mutagenesis. (B) The locations of multiple substitutions that lead to a temperature-sensitive *CDC23* protein are shown. Missense mutations are represented by an arrowhead; nonsense mutation is indicated by a black dot. (C) The specific amino acid substitutions found within the region A of the TPR domain are shown.

blot analysis (Fig. 4, lanes 1 and 2). No signal was observed by using preimmune sera or immune sera presented with excess competing peptide (data not shown). We next used antisera generated against the *TrpE-CDC23* fusion protein to immunoprecipitate *CDC23* from metabolically labelled yeast extracts. A 62-kDa polypeptide that was specific for the immune serum was reproducibly precipitated (Fig. 4, lanes 4 and 5).

The predicted molecular mass of *CDC23* based on conceptual translation of the coding sequence is 73 kDa, considerably larger than the 62-kDa species observed above. It has been shown previously that one other TPR protein, encoded by *nuc2⁺* of *Schizosaccharomyces pombe*, is apparently processed to a smaller form in wild-type yeast cells (12). To assess the possibility of posttranslational processing of *CDC23*, we compared the migration of the *CDC23* gene

FIG. 4. Identification of p62^{cdc23}. Exponential-phase cultures of the yeast strain YPH420 were the source of yeast extracts. Unlabelled extracts were separated by SDS-PAGE, transferred to nitrocellulose, and probed with anti-PEP1 (lane 1) or anti-PEP2 (lane 2). ¹²⁵I-protein A was used for visualization of bound antibody. For immunoprecipitation analysis, total yeast protein was first labelled metabolically with [³⁵S]methionine-cysteine, and then immunoprecipitations were performed by adding control rabbit sera (lane 4) or anti-TrpE-CDC23 sera (lane 5). Full-length CDC23 protein was made in vitro by using a rabbit reticulocyte system and synthetic CDC23 cRNA (lane 6). Lanes 3 and 7 contain the following ¹⁴C-labelled molecular weight standards, myosin, 200,000; phosphorylase b, 97,400; bovine serum albumin, 68,000; ovalbumin, 43,000; carbonic anhydrase, 29,000; β -lactoglobulin, 18,400; and lysozyme, 14,300 (Bethesda Research Laboratories).

product to that of the CDC23 polypeptide produced from the cloned gene in vitro (Fig. 4, lane 6). The largest peptide synthesized in vitro migrated as a 62-kDa peptide by SDS-PAGE, identical in size to the in vivo product. (The smaller

polypeptides in Fig. 4, lane 6, likely represent prematurely terminated versions of CDC23 since they do not immunoprecipitate with anti-PEP2 [data not shown]). The anomalous migration of p62^{cdc23} during SDS-PAGE therefore appears to be an inherent property of the amino acid composition of CDC23.

Immunolocalization of p62^{cdc23} in yeast cells. To determine the intracellular localization of p62^{cdc23}, we performed indirect immunofluorescence on preparations of fixed yeast cells. Figure 5 shows that the antigen recognized by anti-PEP2 is concentrated within the yeast nucleus, coincident with the location of chromosomal DNA. The subcellular location and quantity of p62^{cdc23} do not appear to differ at a gross level between cells at different positions within the cell cycle. Six other TPR proteins, encoded by *nuc2*⁺ (12), *SKI3* (16), *CDC16* (19b), *SSN6* (34), *PRP6* (22), and *cut9*⁺ (42), have been shown to localize to the yeast nucleus as well. The protein product of the more distantly related TPR gene *MAS70* (3) is the only TPR protein to date which targets to a structure outside of the nucleus, the outer mitochondrial membrane (10, 30). Thus, most TPR proteins seem to perform their biological function within the nucleus.

The cdc23 mutant phenotype. Previous studies of *S. cerevisiae* CDC mutants have relied on radioactive labelling of chromosomal DNA and electron microscopy to characterize the stage at which cell division is inhibited (27). We have further examined the *cdc23* terminal arrest phenotype by using the techniques of immunofluorescence microscopy and flow cytometry. At the nonpermissive temperature, cultures of the *cdc23-37* mutant accumulate as a uniform population (>80%) of large-budded cells whose nuclei have migrated to the isthmus separating the two cell bodies (Fig. 6A). Sonication will not separate the two cell bodies at this point. Analysis of these cells using antibodies directed against α and β tubulin reveals a short (preanaphase) mitotic spindle spanning the length of a single nucleus (Fig. 6A). Flow cytometry shows that the bulk of nuclear DNA has been

FIG. 5. Immunolocalization of p62^{cdc23}. Tetraploid yeast cells of the strain 7434-2 were grown in rich medium to exponential phase and fixed with formaldehyde. Affinity-purified anti-PEP2 sera served as the primary antibody, and fluorescein isothiocyanate-conjugated goat anti-rabbit sera served as the secondary antibody (left panel). Nuclear and mitochondrial DNA was visualized by staining with the dye 4',6-diamino-2-phenylindole (DAPI) (right panel). As a control, no staining was observed by using anti-PEP2 and a strain containing *cdc23-47*, an allele which contains a termination codon that effectively removes the PEP2 epitope (data not shown).

FIG. 6. The *cdc23* terminal phenotype. (A) A logarithmically growing culture of a *cdc23-37* mutant strain (YPH945) was shifted from 25 to 36°C for 3 h. Cells were fixed and examined by phase microscopy (left) for DNA staining (center) and for microtubule structure (right). (B) Cell cycle distribution of the genotypes depicted was analyzed by flow cytometry of cultures following shift to 36°C for 3 h. The number of cells is depicted on the vertical axis; fluorescent intensity of emitted light (proportional to DNA content) is shown on the horizontal axis. *CDC23*, YPH500; *cdc23-37*, YPH945.

replicated at this stage of the cell cycle (Fig. 6B). These results are consistent with data from prior studies (27) in demonstrating that *CDC23* function is required for progression beyond a G₂ or early M stage in mitosis.

Eight *cdc23* temperature-sensitive mutant strains (*cdc23-1*, -2, -4, -6, -37, -39, -50, and -54) have been examined for bud and nuclear morphology following a 6-h shift to the nonpermissive temperature. All exhibited >80% uniform arrest morphology (fulfilling the criteria defined for a *cdc* phenotype), except *cdc23-50* and *cdc23-54* which exhibited 73 and 70% terminal arrest morphology, respectively. Thus, mutations in either mutable domain can lead to a *cdc* phenotype.

DISCUSSION

Essential roles for TPR sequences. We have begun to characterize the protein product of the *CDC23* locus of *S. cerevisiae*. Through genetic analysis of deletion and point mutants, we have shown that the tandem TPR units of p62^{*cdc23*} are required for executing this protein's essential mitotic cell cycle function(s). Studies of deletion derivatives of other TPR proteins have arrived at similar conclusions with regard to the essential nature of intact TPR units. Removal of the C-terminal repeats of *nuc2*⁺ by mutagenesis creates a protein that is incapable of complementing a *nuc2* temperature-sensitive allele (13). Extensive mutational analysis of *SSN6* has shown, surprisingly, that all of the *SSN6* polypeptide outside of the repeat block is dispensable for

activity in vivo (34). Truncation of the C terminus of *MAS70* and removal of most of the TPR units result in a polypeptide that is correctly transported to the yeast mitochondria but is nonfunctional (30). Therefore, in all TPR proteins examined thus far, amino acids within the TPR have been implicated in essential functional roles.

Particularly revealing to the essential role of TPR units for *CDC23* function was the inability of an in-frame deletion (*cdc23-Δ5*) that precisely removed one TPR repeat-length unit (TPR-7 to TPR-8) to rescue a *cdc23* null mutation. This mutant polypeptide is clearly expressed, as evidenced by its ability to complement a *cdc23* temperature-sensitive mutation. Intragenic complementation between heteroalleles suggests that *CDC23* polypeptides may interact directly with each other or as part of a supramolecular complex. An alternative mechanism of intragenic complementation, a case in which the sum of two defective activities is more functional than either alone, is possible but unlikely since we have evidence that multiple copies of either the temperature-sensitive or Δ5 mutant is unable to complement the temperature-sensitive growth defect. In this regard, we have used both genetic and physical methods to show that p62^{*cdc23*} associates with at least one other TPR protein, *CDC27*, in vivo (20a, 39). It is possible that TPR protein interactions are mediated by the intra- or intermolecular association of amphipathic α-helices within the TPR segments. Perhaps the different TPR units have differentiated to perform specific functions (or interactions) in conjunction with the mitotic cell cycle. Precedence for such a differentiation phenomenon exists in the human apolipoprotein E, in which one copy of an internal repeat appears to have evolved into the LDL receptor-binding domain (3).

Functional domains within TPR proteins. The amino acid substitutions found in the collection of *cdc23* temperature-sensitive alleles highlight key residues and regions required for *CDC23* function in vivo. In aligning the entire data set of TPR units identified, we have previously noted the existence of three more highly conserved subregions within each repeat (designated A, B, and P [3, 37]). The B and A regions correspond to the "knob" and "hole," respectively, in the three-dimensional model proposed by Hirano et al. (13). Four thermolabile alleles of *cdc23* (-37, -39, -1, and -2) were found to harbor a single amino acid substitution in the A subregion (Fig. 3C). Interestingly, the Ala-to-Thr (*cdc23-37*) and Gly-to-Glu (*cdc23-1*) changes mutate the most highly conserved residue of TPR units, a position which is almost always a small neutral amino acid (Ala, Gly, or Ser). The mutation responsible for the thermolability of the *nuc2-1* mutant in *S. pombe* also maps to the A region of a single TPR unit (13).

The distribution of *CDC23* temperature-sensitive mutations which contain single residue changes is striking in that all of these mutations can be grouped into two distinct mutable domains (Fig. 3A). The C-terminal mutable domain appears to be centered around the most canonical TPR unit (TPR-6) of *CDC23*. It is not clear whether this indicates special significance for this particular repeat unit. The N-terminal mutable domain consists entirely of nonrepetitive amino acids and bears no sequence relationship to other TPR proteins. The existence of two distinct mutable domains raises the possibility that two essential (and perhaps separable) functions are encoded within the *CDC23* polypeptide—one encoded by the TPR units and the other encoded by the unique amino-terminal sequence. This notion is consistent with the proposed evolutionary history of TPR proteins (37). It is interesting that mutations in either domain can result in

TABLE 4. The TPR gene family

Gene	Organism	Approx no. of TPR units	Protein location	Function or phenotype	Reference or source
<i>CDC23</i>	<i>S. cerevisiae</i>	9	Nucleus	Mitosis	37
<i>CDC27</i>	<i>S. cerevisiae</i>	10	ND ^a	Mitosis (?)	36
<i>nuc2⁺</i>	<i>Schizosaccharomyces pombe</i>	10	Nucleus	Mitosis	12
<i>bimA</i>	<i>Aspergillus nidulans</i>	10	ND	Mitosis	26
<i>CDC16</i>	<i>S. cerevisiae</i>	10	Nucleus	Mitosis	16
<i>cut9⁺</i>	<i>Schizosaccharomyces pombe</i>	10	Nucleus	Mitosis	Samejima and Yanagida, unpublished data
<i>SSN6</i>	<i>S. cerevisiae</i>	10	Nucleus	Transcription	33
<i>SK13</i>	<i>S. cerevisiae</i>	8	Nucleus	Transcription	29
<i>PRP6</i>	<i>S. cerevisiae</i>	9	Nucleus	Splicing	22
<i>STI1</i>	<i>S. cerevisiae</i>	8	ND	Stress induced	25a
<i>IEP SSP 3521</i>	<i>Homo sapiens</i>	8	Nucleus, Golgi apparatus, small vesicles ^b	Heat shock and trans-formation sensitive	15
<i>MAS70</i>	<i>S. cerevisiae</i>	7	Mitochondria	Protein import	10
<i>MOM72</i>	<i>Neisseria crassa</i>	7	Mitochondria	Protein import	40
ORF 3' <i>recA</i>	<i>Synechococcus</i> sp.	5+	ND	Unknown	25
<i>cm</i>	<i>Drosophila melanogaster</i>	16	ND	Neurogenesis	Smouse and Perrimon, unpublished data

^a ND, not determined.^b Present mainly in the nucleus in simian virus 40-transformed fibroblasts and in the Golgi apparatus and small vesicles in their untransformed counterparts (15).

a uniform terminal arrest morphology (*cdc* phenotype) at the nonpermissive temperature.

Function(s) of TPR proteins. To date, the exact biochemical function of *CDC23* and all TPR proteins remains unknown. Immunolocalization studies of several TPR members has demonstrated that all but *MAS70* are concentrated diffusely within the nucleus (Table 4). It has been suggested that *nuc2⁺* may serve as a component of the yeast nuclear scaffold because of the proteins relative insolubility in certain detergents (13). The biological significance of this in vitro finding remains to be demonstrated. The TPR protein *SSN6* was originally isolated as a genetic locus required for repressing glucose-repressible transcription units under specific culture conditions (33). Studies using hybrid *SSN6-LEXA* proteins have established that *SSN6* can act directly to repress transcription if appropriately targeted to a yeast promoter (18). To date, several lines of investigation have failed to demonstrate direct DNA binding by *SSN6* (3a), perhaps indicating that *SSN6* functions as only one component of a transcriptional-repression complex. The observation that *SSN6* has structural features of bona fide DNA-binding transcriptional regulators (3) in addition to TPR units is consistent with the proposed modular architecture of TPR proteins described above.

Future experiments involving the TPR proteins must be directed toward elucidating their biochemical functions. Genetic analysis of *trans*-acting suppressors may serve as a means to link TPR proteins with genes of known function. Temperature-sensitive mutations that map within TPR units, such as those described here for *CDC23*, may be particularly useful for identifying interacting proteins. Toward this goal, we have recently produced a collection of *cdc23* suppressors, many of which exhibit the phenotypes of chromosome instability and temperature sensitivity (38a). Characterization of these suppressors may provide clues necessary for a molecular understanding of *CDC23* and TPR function.

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